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Cell Proliferation and Apoptosis in Normal Liver and Preneoplastic Foci

by Rolf Schulte-Hermann[1], Wilfried Bursch[1], Bettina Kraupp-Grasl[1], Franziska Oberhammer[1], Alexandra Wagner[1], and Randy Jirtle[2]

The growth rate of tissues including tumors is determined by the difference between cell replication and cell death. Among different types of cell death, apoptosis, a form of programmed cell death, is of particular importance. Nongenotoxic carcinogens exert their carcinogenic effects not only via stimulation of cell replication but also by modulating the incidence of apoptosis. This can be seen at different stages of carcinogenesis: *a*) After initiation in the liver, many initiated cells may undergo apoptosis and never develop into preneoplastic foci, as suggested by both biological and mathematical studies. Thus, apoptosis appears to determine the efficiency of initiation. *b*) In the promotion stage, early preneoplastic hepatic foci originate either from treatment with a genotoxic carcinogen or spontaneously exhibit much higher rates of cell replication than normal cells, but nevertheless show little preferential growth. This is due to enhanced rates of apoptosis. Some tumor promoters were found to inhibit apoptosis and thereby accelerate foci growth and carcinogenesis. *c*) In neoplastic nodules and tumors, apoptosis has been shown to be an important growth determinant and to be regulated by growth regulatory hormones, which thereby may decrease or accelerate tumor growth. Studies on the regulation of apoptosis revealed that in the liver, transforming growth factor TGF-β_1 is involved in the initiation of apoptosis. This was based on three lines of evidence: TGF-β_1 induced apoptosis in isolated hepatocytes, *b*) *in vivo* hepatocytes undergoing apoptosis showed positive immunostaining with antibodies against a precursor of TGF-β_1. This staining response was not seen in normal or necrotic hepatocytes, *c*) injection of TGF-β_1 into intact animals induced apoptosis in the liver *in vivo*.

Introduction

Many seemingly nongenotoxic compounds produce tumors in rodent liver, some of which (e.g., certain steroids) have also been associated with tumor formation in human liver (*1,2*). A common denominator of the actions of many if not all of these compounds is a pleiotropic effect on rodent liver consisting of organ enlargement and hyperplasia associated with functional changes such as increases in activities of certain cytochromes P450, of other drug-metabolizing enzymes, or of enzymes involved in fatty acid metabolism (*1,3,4*). Typically, these agents induce an initial burst of enhanced cell proliferation, which disappears after a few days, despite further treatment. This burst results in the development of hyperplasia, which usually is sustained as long as treatment lasts. In fact, as in other systems where nongenotoxic carcinogens or tumor promoters have been studied, sustained hyperplasia seems to be a common denominator.

When treatment is stopped, a rapid regression of hyperplasia may occur, depending on the compound. This regression in the liver is due to a steep increase in the rate of apoptosis. (*5*). Apoptosis is a process whereby organisms can actively eliminate excessive or damaged cells (*6*). In the liver and other organs, apoptosis is complementary to mitosis, maintaining adequate cell numbers. Apoptosis was originally defined by Kerr et al. on morphological and functional grounds (*6*). Up to now, morphological characteristics (particularly when light and electron microscopy are

[1]Institute of Tumorbiology-Cancer Research, University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria.
[2]Department of Radiology, Duke University, Box 3433, Durham, NC 27710.
Address reprint requests to: R. Schulte-Hermann, Institute fur Tumorbiologie-Krebsforschung, A-1090 Wien, Borschkegasse 8a, Austria.
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Table 1. Inhibition of apoptosis by tissue-specific mitogens.

Tissue	Mitogen	Reference
Adrenal cortex	ACTH	(*6*)
Liver	CPA, PB, α-HCH, Naf	(*7*)
Liver preneoplasia	PB, CPA	(*7*)
Prostate	Testosterone	(*25*)
Neuronal cells	NGF	(*26*)
Thymocytes	TPA	(*27*)
Estrogen-dependent Kidney tumor	DES	(*17*)

Abbreviations: ACTH, adrenocorticotrophic hormone; CPA, cyproterone acetate; PB, phenobarbital; HCH, hexachlorocyclohexane; NGF, nerve growth factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; DES, diethylstilbestrol.

combined) and functional criteria are the most reliable markers of apoptosis.

Among the functional characteristics, inhibition of apoptosis by tissue-specific growth factors and mitogens is of great interest (Table 1). Inhibition may be used to discriminate apoptosis from necrosis (*5–7*) and supports the regulatory role of apoptosis in tissue homeostasis.

Apoptosis has generally been assumed to be a rapid process; recently a direct estimation in the liver revealed that the duration of the histologically visible stages in this organ is approximately 3 hr (*8*).

Apoptosis does not appear to occur at random in all hepatocytes. During regression of mitogen-induced hyperplasia, nonproliferating (old) hepatocytes seemed to be preferred for apoptosis. Furthermore, studies on apoptosis in putative preneoplastic foci of rat liver have shown that foci cells exhibit about 10-fold higher rates of apoptosis than normal hepatocytes (*7,12*). The cellular and molecular basis of this apparent selectivity of apoptotic mechanisms is not known.

Role of Cell Replication and Apoptosis in Multistep Chemical Carcinogenesis

It is generally accepted that cell proliferation plays an important role at different stages of cancer development. During initiation, cell replication is considered to "fix" genetic insults. Promotion includes amplification of initiated cells and therefore by definition depends on cell proliferation. Multiple studies supporting the importance of cell proliferation have been published and reviewed (*1,9–12*); they will not be discussed here.

Apoptosis may also have important roles in different stages of carcinogenesis. We described some years ago that putative preneoplastic liver cells not only show higher replicative activity than normal liver cells but also enhanced rates of apoptosis which may balance proliferation so that no net growth results (*7,12*). Recently, Moolgavkar et al. (*13*), on the basis of mathematical calculations of foci growth rates, predicted that initiated cells can be extinguished. Columbano et al. (*14*) provided the first experimental evidence supporting this prediction. They showed that initiating doses of various genotoxins do not produce stable initiation in a hyperplastic liver undergoing massive regression through apoptosis.

We recently tried to quantify initiated cells in the early stages of cancer development, namely in the first days after the cytotoxic dose of the initiating carcinogen *N*-nitrosomorpholine (NNM). Glutathione-S-transferase (GST-P) was used as a marker as described by Ito (*15*). Coinciding with regenerative DNA synthesis, there was a dramatic increase of GST-P positive single cells and mini-foci, which after a plateau declined and finally reached approximately 20% of the peak value. Although there is no absolute proof that GST-P positive cells are truly initiated and that they only disappear through apoptosis, these observations fit well the mathematical predictions (*13*). A similar decline of GST-P positive cells has also been observed by Satoh et al. (*16*) in a study with diethylnitrosamine. Thus, it seems likely that initiated cells can be eliminated under certain experimental conditions, and the old dogma that initiation is irreversible may need some modification in the future.

When investigating the promotion stage after NNM treatment, we noted two apparent paradoxes. The first one was that putative preneoplastic foci exhibited much higher cell proliferation than normal liver but showed almost no net growth. We then found high apoptotic activity in foci, which apparently balanced enhanced cell replication (*7*). The second paradox was that during tumor promotion by phenobarbital (PB), a rapid expansion of phenotypically altered foci occurred without a persistent increase of cell proliferation. The explanation was that PB (and other tumor promoters) can inhibit apoptosis in foci (*7*). The enhanced apoptotic activity was associated with an apparent phenotypic instability of the foci, which may reflect the situation in the normal tissue where apoptosis occurs during down regulation of the pleiotropic response (*12*).

Apoptosis also occurs in later stages of carcinoma development, namely, in neoplastic nodules and malignant tumors (*6*). Some observations have shown that tumor cells may depend on the presence of trophic hormones (*17,18*). Using an estrogen-dependent transplantable hamster kidney tumor, we recently observed that withdrawal of diethylstilbestrol (DES) enhanced apoptosis and decreased mitosis, whereas treatment with DES had the opposite effects on both events (*17*). It appears therefore that both cell replication and cell death by apoptosis are regulated in concert to produce regression or growth of organs and that these basic mechanisms are still functioning in malignant tumors, although they are obviously out of balance. Chemicals may shift the delicate balance between replication and death of cells (Fig. 1). The roles of apoptosis in the stages of carcinogenesis are summarized in Table 2.

In conclusion, for understanding chemically induced

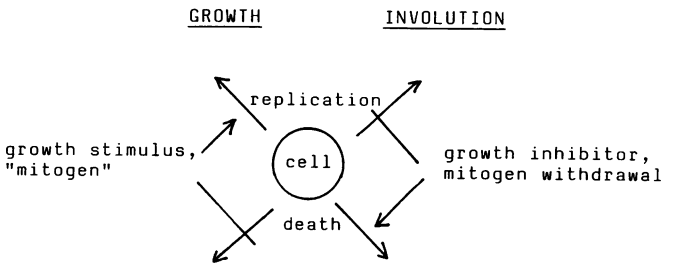

FIGURE 1. Regulation of growth and involution of tissues.

Table 2. Importance of apoptosis in the stepwise development of cancer.

Initiation	Efficiency determined by death/disappearance of initiated cells. Can initiation be reversible?
Promotion	Clonal expansion favored by inhibition of apoptosis by tumor promoter. Reversibility of promotion by enhanced apoptosis after promoter withdrawal.
Tumor growth	Rate determined by balance between cell replication and cell death. Apoptosis can be increased by withdrawal of trophic hormones or by treatment with antihormones.

carcinogenesis and for evaluating health risks, studies restricted to single phenomena such as mutagenesis, mitogenesis, cell death, or others are not sufficient. It is essential that all factors are taken into account and that an attempt is made to understand the complex interplay of chemicals and processes at the various stages of carcinogenesis. It should also be pointed out in this context that the term mitogen can be highly misleading when its possibly inherent potential to inhibit cell death is not taken into account. We therefore recommend restriction of the term mitogen to situations where mitotic events are addressed and use of the term growth stimulus in less specific situations.

TGF-β_1 As a Signal Regulating Apoptosis

Obviously, understanding the regulation of initiation of apoptosis is important for elucidating mechanisms of chemical carcinogenesis. As a first candidate, we selected transforming growth factor TGF-β_1, which is associated with negative growth control in the liver and in some other epithelial tissues (*19*). Furthermore, some peptides related to TGF-β_1 such as anti-Mullerian hormone have already been shown to induce regression of tissues.

We found that cells undergoing apoptosis in the liver *in vivo* show immunostaining with antibodies against an epitope of the precursor molecule of TGF-β_1, whereas normal or necrotic hepatocytes were not stained (*20*). Furthermore, there were a few isolated, intact hepatocytes that reacted with the antibodies, and their incidence correlated with the incidence of apoptosis in the liver. These cells were smaller, suggesting condensation of the cytoplasm, an early feature of apoptosis. These findings suggest that hepatocytes preparing for apoptosis may either synthesize TGF-β_1 or take it up selectively from neighboring cells in its precursor form. In any event, the precursor of TGF-β_1 or its breakdown products persist in the cytoplasm beyond the point where protein synthesis and intercellular exchange cease in apoptotic hepatocytes (*20*).

To test whether TGF-β_1 would actually be able to induce apoptosis, we added it to cultures of isolated hepatocytes or injected it *in vivo*. In both situations, a striking increase of apoptosis could clearly be demonstrated. Furthermore, it was found that a putative precursor of TGF-β_1, the latency-associated peptide (obtained from Dr. A. Purchio, Seattle, WA) was less active to induce apoptosis (*20,22*). This suggests that TGF-β_1 in its mature form is a trigger for apoptosis in hepatocytes.

Cell death by apoptosis is frequently associated with endonuclease activation and formation of a characteristic DNA fragment pattern (DNA ladder) resulting from oligonucleosomes (*23*). This has led some authors to hypothesize that endonuclease activation is an early and necessary step of apoptosis. In contrast, we have not been able to demonstrate endonuclease activation early during apoptosis in TGF-β_1-treated hepatocytes (*24*). Therefore, the characteristic DNA fragmentation (ladder formation) does not appear to be a phenomenon generally occurring during apoptosis, at least not as one of the initial steps. Ladder formation should not be used as the sole marker of apoptosis.

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